



## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

B26

(51) International Patent Classification 6: <b>C07H 21/04, C12Q 1/68</b>		A1	(11) International Publication Number: <b>WO 95/18818</b>
			(43) International Publication Date: <b>13 July 1995 (13.07.95)</b>
(21) International Application Number: <b>PCT/US95/00158</b>		(81) Designated States: AU, CA, JP, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).	
(22) International Filing Date: <b>3 January 1995 (03.01.95)</b>			
(30) Priority Data: <b>08/177,241 3 January 1994 (03.01.94) US</b>		Published <i>With international search report.</i>	
<p>(71) Applicant: CHILDREN'S HOSPITAL OF PHILADELPHIA [US/US]; 34th Street and Civic Center Boulevard, Philadelphia, PA 19104 (US).</p> <p>(72) Inventor: FELIX, Carolyn, A.; 118 Valley Road, Ardmore, PA 19003 (US).</p> <p>(74) Agents: JOHNSON, Philip, S. et al; Woodcock Washburn Kurtz Mackiewicz &amp; Norris, 46th floor, One Liberty Place, Philadelphia, PA 19103 (US).</p>			
<p>(54) Title: <b>POLYMORPHISM AT CODON 36 OF THE p53 GENE</b></p> <p>(57) Abstract</p> <p>A polymorphism at codon 36, exon 4, of the p53 gene has been identified. Methods for ascertaining genetic linkage of the polymorphism with germline mutations in p53 which lead to tumor development, for ascertaining parental origin when such linkage is ascertained, and for determining familial inheritance are provided.</p>			

**FOR THE PURPOSES OF INFORMATION ONLY**

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	GB	United Kingdom	MR	Mauritania
AU	Australia	GE	Georgia	MW	Malawi
BB	Barbados	GN	Guinea	NB	Niger
BE	Belgium	GR	Greece	NL	Netherlands
BF	Burkina Faso	HU	Hungary	NO	Norway
BG	Bulgaria	IE	Ireland	NZ	New Zealand
BJ	Benin	IT	Italy	PL	Poland
BR	Brazil	JP	Japan	PT	Portugal
BY	Belarus	KE	Kenya	RO	Romania
CA	Canada	KG	Kyrgyzstan	RU	Russia Federation
CF	Central African Republic	KP	Democratic People's Republic of Korea	SD	Sudan
CG	Congo	KR	Republic of Korea	SE	Sweden
CH	Switzerland	KZ	Kazakhstan	SI	Slovenia
CI	Côte d'Ivoire	LI	Liechtenstein	SK	Slovakia
CM	Cameroon	LK	Sri Lanka	SN	Senegal
CN	China	LU	Luxembourg	TD	Chad
CS	Czechoslovakia	LV	Latvia	TG	Togo
CZ	Czech Republic	MC	Monaco	TJ	Tajikistan
DE	Germany	MD	Republic of Moldova	TT	Trinidad and Tobago
DK	Denmark	MG	Madagascar	UA	Ukraine
ES	Spain	ML	Mali	US	United States of America
FI	Finland	MN	Mongolia	UZ	Uzbekistan
FR	France			VN	Viet Nam
GA	Gabon				

## POLYMORPHISM AT CODON 36 OF THE P53 GENE

Field of the Invention

This invention relates to screening methods based upon the identification of a polymorphism at codon 36, in exon 4, of 5 the p53 gene.

Background of the Invention

The p53 gene on chromosome 17, specifically region 17p, is the most commonly altered gene in human cancer. Rearrangements of the gene resulting in inactivation have been 10 observed in human and animal cell lines and tumors. The wild type gene product is hypothesized to function in tumor suppression. Consequently, there has been considerable interest in detecting mutations in this gene in tumors. A number of studies have already reported mutations in tumors of 15 diverse origin. Most of the mutations which have been detected have been detected in exons 5-9 of the gene. It has also been reported that transfection of the wild-type p53 arrests the growth of cancer cell lines. (Baker, et al., *Science*, 249:912-915 (1990); Friedman, et al., *Proc. Natl. Acad. Sci. USA*, 20 87:9275-9279 (1990)).

Most of the p53 mutations identified are "missense" mutations resulting in a single amino acid change. In a recent review article, it was reported that 83% of the p53 mutations are such missense mutations. Harris, et al., "Clinical 25 Implications of the p53 Tumor-Suppressor Gene," *The New England Journal of Medicine*, 329:1318-1327 (1993) (incorporated herein by reference).

One problem hindering the identification of mutations in the p53 gene is the occurrence of sequence polymorphisms, 30 some of which are restriction fragment length polymorphisms

- 2 -

(RFLPs), in this same gene. Comparison with normal tissue from a patient having a tumor exhibiting a purported mutation in p53 must be made to verify whether the mutation is somatic or germline in origin. Another possibility is that the change in 5 the germline is a polymorphism. Silent mutations in the germline can be indistinguishable from a polymorphism, unless recognized as such in larger population based studies.

Analysis of germline tissues from patients is not always feasible, as many diagnoses are made retrospectively. 10 Thus, the identification of polymorphisms in this gene can aid interpretation of results from tumors.

Several polymorphisms have already been identified in p53, but only three others are in the coding sequence--the rest are in introns. A continually updated compendium will aid in 15 diagnostic interpretation. Discussion of the identified polymorphisms follows.

Ahuja, et al., "Variation in the protein coding region of the human p53 gene," *Oncogene*, 5:1409-1410 (1990) report a polymorphism at residue 21 of the p53 gene in the vicinity of 20 exon 2. The polymorphism was only identified in 1 of 106 samples analyzed and was identified using *Bgl*III. The authors report that the rarity of their polymorphism limits its usefulness in studies of allelic loss.

Ara, et al., "Codon 72 polymorphism of the TP53 gene," 25 *Nucleic Acids Research*, vol. 18, no. 16, 4961 (1990) report a polymorphism at residue 72, exon 4, of the p53 gene. The polymorphism results in an amino acid residue alteration and can be identified using *Acc*II. The frequency of the polymorphic allele is reported as 0.64. The frequency of 30 heterozygosity is not reported, nor is it ascertainable from the reference.

In de la Calle, et al., "Siallelic *Bgl* II DNA polymorphism of the human p53 oncogene," *Nucleic Acids Research*, vol. 18, no. 1, p. 206 (1990), the authors report a 35 polymorphism somewhere in nucleotides 1 to 1587. The polymorphism can be identified using *Bgl*III. The frequency of the polymorphic allele is reported as 0.05.

- 3 -

In de la Calle-Martin, et al., "MspI polymorphism of the human p53 gene," *Nucleic Acids Research*, vol. 18, no. 16, 4963 (1990), a polymorphism in the coding region of p53 is reported upon. The polymorphism can be identified using *MspI*.

5 The frequency is reported as 0.72.

In de la Calle-Martin, et al., "AccII polymorphism of the p53 gene," *Nucleic Acids Research*, vol. 18, no. 16, 4963 (1990), the authors report a polymorphism in exon 4 of p53. The polymorphism can be identified using *AccII*. The frequency 10 is reported as 0.68.

Chumakov, et al., "BstNI/NciI polymorphism of the human p53 gene (TP53)," *Nucleic Acids Research*, vol. 19, no. 24, 6969 (1991) report a polymorphism within the 6th intron of the human p53 gene that can be revealed using restriction 15 nuclease digestion. One allele is cleaved by *BstNI*, the other by *NciI*. Allele frequencies are reported as 0.31 and 0.69, respectively. Prosser, et al., "Biallelic ApaI polymorphism of the human p53 gene (TP53)," *Nucleic Acids Research*, vol. 19, no. 17, 4799 (1991) report a polymorphism in 20 intron 7 of p53. The polymorphism can be identified using *ApaI*. The expected frequency of heterozygosity is reported as 10.3 %. The authors suggest that the polymorphism can be used to detect loss of a p53 allele in tumor material.

Carbone, et al., "Polymorphism at codon 213 within the 25 p53 gene," *Oncogene*, 6:1691-1692 (1991) report a polymorphism at codon 213, exon 6, of the p53 gene. The polymorphism was detected in 6 cases and results in the loss of a *TagI* site. The authors report that the change would be silent. The authors also report that at least two true somatic mutations 30 alter the same restriction site.

Mazars, et al., "Nucleotide sequence polymorphism in a hotspot mutation region of the p53 gene," *Oncogene*, 7:781-782 (1992) also report a polymorphism in codon 213, in the last base. The authors noted that this is a "hotspot" mutation 35 region. The polymorphism does not change an amino acid. The polymorphism was identified using *SSCP*. The frequency varied depending upon whether the tissue was of normal or tumor

- 4 -

origin. The highest frequency was observed in a breast cancer patient population. As a result of this observation, the authors attempted to determine whether there was any association with familial breast cancer. None was shown.

5        Willems, et al., "PCR detection of a *Bgl*II polymorphism in intron I of the human p53 gene (TP53)," *Nucleic Acids Research*, vol. 20, no. 5, 1172 (1992) report a polymorphism in intron I of p53. The polymorphism can be identified using *Bgl*II. The frequency is reported as 0.08.

10        As already noted above, knowledge of all possible polymorphisms in the p53 gene will facilitate identification of mutations. However, distinguishing between polymorphisms and germline mutations requires larger population based studies. Knowledge of a specific single polymorphism can be used to 15 eliminate a mutation in p53 as a causative factor in a tumor if the purported mutation displays the same pattern as the polymorphism. In addition to this application, knowledge of a specific single polymorphism can facilitate the determination of the genetic linkage of an identified mutation and, 20 therefore, the tracing of parental origin and familial histories without requiring laborious sequence determination if the mutation is of germline origin. It should be noted, however, that some germline mutations could arise *de novo*. Genetic linkage of presently unidentified genes distinct from 25 p53 for which loss of heterozygosity has been linked with cancer or other diseases is also facilitated. Additionally, knowledge of a specific single polymorphism can facilitate determination of loss of heterozygosity. This is particularly important due to the occurrence of loss of heterozygosity of 30 p53 in tumorigenesis. An association between a p53 mutation and loss of heterozygosity on chromosome 17p has been shown in tumors. *Harris, et al., supra.*

Not all polymorphisms are of equal utility in the aforementioned applications. As a mutation leading to tumor 35 development is most likely to occur in the coding region, a polymorphism in the coding region, i.e. an exon, is preferable. These regions of the gene are most often studied.

- 5 -

Polymorphisms in the coding region are generally less common as the coding regions of the gene are more stable and less mutable over time. Further, a coding region polymorphism enables screening of both genomic and cDNA. It is also preferable that 5 the polymorphism result in a change in a restriction enzyme site.

For purposes of genetic linkage determinations, it is further preferable that the polymorphism not occur in the same sites where somatic mutations are known to occur most 10 frequently. Four "hot-spots" in p53 frequently altered in human cancers are reported in Nigro, et al., *Nature*, 342:705-708 (1989) (incorporated herein by reference). These hot spots occur in the conserved regions of the gene. Also, it is preferable that somatic mutations not result in the same 15 detectable change as the polymorphism, but this is a possibility.

For determinations of parental origin, it is preferable that the occurrence of the polymorphism is not too frequent. Preferably, the frequency of heterozygosity should 20 be high enough to be informative, but low enough such that it is not often found in both parents. The homozygous state for the polymorphism is very rare.

The present inventor has identified a polymorphism in codon 36 of exon 4 of the p53 gene which meets the foregoing 25 criteria. It involves the change of a *F*nu*I* restriction site to a *B*pu*I* site. Although the specific codon change was reported upon previously in Chiba, et al., *Oncogene*, 5:1603-1610 (1990), Chiba et al. reported that the change was a silent mutation in non-small cell lung cancer, not a polymorphism. Chiba et al. 30 do not disclose that the mutation was confirmed as being somatic, nor do they report testing which would confirm whether or not the observation, if not somatic, was a silent germline mutation or polymorphism. For reasons presented above, whether an observed germline mutation is in fact a mutation or a 35 polymorphism is an important difference. The present inventor discovered that the change can be due to a polymorphism. Although the change is "silent" in terms of amino acid coding,

- 6 -

the change does result in the change from a *Fni*I restriction site to a *Bcc*I restriction site. This was not recognized previously.

5 Summary of the Invention

In one aspect, the present invention involves a method for determining genetic linkage of presently unidentified genes in a region of chromosome 17 for which loss of heterozygosity has been linked with cancer or other diseases. This involves 10 mapping of the region of genomic loss with respect to other markers in the region to which the gene of interest can be linked. The polymorphism at codon 36 of p53 is one such marker.

In another aspect, the present invention relates to 15 a method for determining whether a germline mutation in p53 phenotypically expressed as a tumor in a patient is linked to the polymorphism at codon 36, exon 4, of the p53 gene. The method involves isolation of genomic DNA from the tumor of a patient, amplifying the regions corresponding to the mutation 20 and exon 4 of p53 if necessary, identifying the polymorphism in the amplified sample, cloning the amplified products containing the mutation and the polymorphism, sequencing the cloned products, and determining whether the mutation and polymorphism occur in the same clone. Such occurrence indicates the 25 mutation is on the same allele. If not, the mutation is on a different allele. In a further aspect, the present invention involves a method for determining parental origin of an allele carrying a germline mutation in p53. The method involves screening tissue from the parents of a patient 30 exhibiting a tumor with a germline mutation which has been linked to said polymorphism for identification of the parental source of the polymorphism.

In yet another aspect, the present invention involves a method for determining familial histories and, therefore, the 35 relation of the polymorphism, if any, to tracking of the mutation and predilection for tumor development. The method involves screening tissue from relatives of a patient

- 7 -

possessing an allele carrying the mutation for presence of the polymorphism.

In another aspect, the present invention involves a method for determining loss of heterozygosity.

5 Brief Description of the Drawings

Figure 1 depicts the occurrence of the polymorphism in gangliocytoma/ganglioneuroma, neuroblastoma, and normal specimens.

Figure 2 depicts the localization at codon 36 by sequence 10 determination.

Detailed Description of the Invention

Mutations in p53 have been linked with a variety of cancers. Mutations can be somatic or germline in origin, the distinction being that germline mutations are present in all 15 cells of the body, tumor or otherwise. Many of the mutations observed in p53 are somatic in origin. However, germline mutations, when identified, can be significant due to the possibility they represent genetic inheritance. Germline mutations, however, can also be *de novo*, i.e. originate during 20 embryogenesis. Although inherited mutations are much less common, they have been identified in family cancer syndromes such as the Li-Fraumeni syndrome. Li et al., "Rhabdomyosarcoma in children," *J. Natl. Cancer Inst.*, 43:1365-1373 (1969). The potential for inherited predilection to cancer development 25 increases the significance and desirability of screening methods

for rapidly and sensitively determining inheritance.

The present invention relates to the identification of a polymorphism at codon 36 in exon 4 of the p53 gene. Exon 30 4 is the largest exon of the p53 gene and spans from codons 22-125. Although exon 4 does not contain a mutational "hotspot", others have reported mutations in this region. Caron de Fromental, et al., *Genes Chrom. Cancer*, 4:1-15 (1992). An

example of a mutational "hotspot" is the region spanning amino acids 130 to 240. A number of point mutations were identified in this region, which led to significant, tumor-promoting changes in phenotype. WO 92/00311, published January 9, 1992.

5 See also Nigro, et al., *supra*.

Polymorphisms provide an opportunity for screening methods upon establishment of linkage with a particular mutation. Although the mutations themselves can be screened, the polymorphism may be a restriction fragment length 10 polymorphism, resulting in the change in length of fragments cleaved with a particular enzyme, such that the presence of the polymorphism can be readily ascertained without resort to sequencing or PCR. Upon establishment of linkage with a mutation, an RFLP allows for simpler analysis, such as in the 15 establishment of pedigrees.

Additionally, polymorphisms allow for the linkage determination of genes which have not yet been specifically identified, but have been identified in that a loss of heterozygosity in a region purportedly containing the gene 20 results in the development of cancer or other disease conditions. Thus, pedigree analysis of unidentified genes is also possible. One method used for screening for mutations is single-strand conformation polymorphism (SSCP) analysis. A point mutation in a small DNA fragment causes a significant 25 conformational change which is detectable as a difference in mobility in non-denaturing acrylamide gel electrophoresis. A limitation of this method is that it does not distinguish between polymorphisms and mutations. The initial identification of polymorphisms by SSCP, or other methods, 30 followed by characterization by sequencing, should obviate the need for further sequencing when the same polymorphic patterns are found in other cases. Similarly, knowledge of a single specific polymorphism can be helpful. See, for example, the discussion in Mitsudomi, et al., *supra* regarding the purported 35 mutations at codon 213. During screening, a polymorphism at codon 213 was noticed. To exclude the polymorphism, side by side comparisons with a control sample using SSCP were

- 9 -

performed. This pattern comparison revealed that 4 of 13 purported mutations in exon 6 may have been polymorphisms.

Various methods are available in the art for identification of mutations in the p53 gene. For example, see 5 WO 92/13970, published August 20, 1992; WO 92/00311, discussed previously; WO 93/20233, published October 14, 1993; and EP 0390323, published October 3, 1990 (subject matters of which are incorporated herein by reference). Mutations can be determined using SSCP or denaturing gradient gel 10 electrophoresis (DGGE) as PCR-based methods. If the mutation changes a restriction site, or results in a large deletion, the mutation can also be initially recognized by Southern blot.

The present invention is not limited to any one particular manner of identifying the mutation. Of course, 15 mutations can also be identified through the more laborious task of sequencing entire gene isolates or genomic hot spots.

Mutations which have been causatively linked to tumor development and/or progression are preferable. That is, tissues of patients displaying mutations which result in 20 phenotypic expression of tumors are preferred for subjecting to the methods according to the invention. However, as many mutations lead to a pre-cancerous state and depend upon subsequent mutations in the sister allele for entering the cancerous state, such mutations are also contemplated, as they 25 provide a mechanism for earlier diagnosis. See discussion in WO 92/00311 discussed, *supra*. In WO 92/00311, a pre-cancer cell is defined as a cell that has one normal p53 allele and one mutated p53 allele. In a cancer cell, both alleles are mutated. If the present polymorphism is linked to a mutation, 30 it may be possible to identify this pre-cancer state. However, it is also possible that allelic loss may precede mutation in the sister allele. Such is the case with rhabdomyosarcoma (RMS). Felix, et al., *Cancer Research*, 52: 2243-2247 (1992) (incorporated herein by reference).

35 Once a mutation has been identified, the method according to the invention can be utilized to determine linkage of the mutation with the polymorphism. Linkage determinations

- 10 -

can provide numerous diagnostic advantages. For example, once linkage is established, allelic loss can also be followed. As discussed above, linkage determinations can also be useful for 17p loci other than p53--distal 17p loci are suspected to be 5 involved in brain tumors. Thus, with the establishment of a linkage, it is then possible to screen for carriers.

Further, once a linkage has been determined, parental origin of the allele carrying the mutation can be ascertained. This could enable back-tracking to determine familial 10 inheritance if the mutation is inherited. As discussed previously, however, not all germline mutations are inherited. Additionally, forward-tracking can be utilized to identify carriers. In this manner, siblings and offspring can be screened for the same polymorphism. In the methods according 15 to the invention, amplification of the DNA region containing the mutation is contemplated if the sample is limited. Amplification can be performed by the technique of polymerase chain reaction (PCR) such as disclosed in U.S. Patent No. 4,683,194 issued to Saiki et al (incorporated herein by 20 reference). Primers directed to the coding sequence of p53, or to a specific exon within the coding sequence, can be utilized. For an example of primers directed to exon 4, see Mitsudomi et al., *Oncogene*, 7:171-180 (1992), incorporated herein by reference. The primers used in Example 1 below were designed 25 by Mitsudomi. The PCR products are then cleaved with restriction enzymes, size separated on agarose gels and the polymorphism identified. The polymorphism can also be identified by SSCP.

Alternatively, the polymorphism can be identified by 30 Southern blot, using restriction enzymes *BccI* and *FinI* run in parallel. Fragment patterns can be compared with a standard containing the polymorphism also subjected to cleavage with the enzymes or with the pattern disclosed herein in Figure 1. DNA isolated from a sample known to contain the polymorphism 35 can be simultaneously subjected to the same amplification and cleavage. If the polymorphism is detected in the sample, genetic linkage can be confirmed by sequencing.

- 11 -

Upon the establishment of genetic linkage, screening to determine parental origin of the polymorphism provides insight into the potential hereditary origin of the mutation and, hence, the tumor. Parental origin screens can be 5 performed in a manner similar to screening the p53 gene, or region thereof, for possession of the polymorphism. Rather than being performed upon tissue extracted from a tumor, however, such screens are performed upon normal tissue isolated from parents and the patient. The same procedure as described 10 above is then followed. The heterozygous occurrence of this polymorphism is infrequent enough that it is unlikely that both parents would be heterozygous.

A similar procedure as outlined above is performed in order to determine familial carriage of distal related genes, 15 especially when those genes have not been isolated. The presence of the polymorphism in a sibling or offspring might indicate proclivity for the mutation and, hence, tumor development.

#### Materials and Methods

##### 20 Oligonucleotide Primers

The primers used for amplification were obtained from Dr. Mitsudomi. See Mitsudomi et al., *supra*. The primers were derived from intron 3 and intron 4 of p53. The sequences of the primers are as follows:

25 5'AGGACCTGGTCCTCTGACTC 3'      intron 3      SEQ ID NO:1  
3'CATTGAAGTCTCATGGAAGC 5'      intron 4      SEQ ID  
NO:2.

##### Single-strand conformation polymorphism (SSCP)

Point mutations in the p53 gene were detected using 30 a modified version of the PCR/SSCP method (Orita et al., *PNAS USA*, 86:2766-2770 (1989) and *Genomics*, 5:874-879 (1989). Suzuki et al., *Oncogene*, 5:1037-1043 (1990), (incorporated herein by reference. One hundred nanograms of genomic DNA was amplified in a volume of 10  $\mu$ l containing 50 mM potassium

- 12 -

chloride, 10 mM Tris-HCl (pH 8.3), 1.5 mM magnesium chloride, 0.01% (w/v) gelatin, 1.25 mM each of four dNTPs (Pharmacia), 0.05 µg of the pair of primers, 0.25 units of Taq DNA polymerase (Perkin Elmer Cetus, Norwalk, CT, USA) and 0.5 µl of 5 [α-<sup>32</sup>P]dCTP (300 Ci/mmol<sup>-1</sup>, 10mCi/mmol<sup>-1</sup>, Amersham, Arlington, IL, USA). The amplification reaction using a thermal cycler (Perkin-Elmer Cetus) consisted of 94°C for 9 minutes for initial denaturation when using genomic DNA, followed by 35 10 cycles of 94°C for 1 minute, annealing for 1 minute at 55°C (genomic DNA), extension at 72°C for 2 minutes, and a 7 minute final extension at 72°C. One µl was then cleaved with the enzyme AlwNI in 10 µl to reduce the fragment sizes to those more suitable to SSCP.

Two micro-liters of the digested product was 15 transferred to a 96 well plate, mixed with loading buffer (95% formamide, 20 mM EDTA, 0.05% xylene cyanol, 0.05% bromophenol blue) and incubated in a 90°C water bath for 5 minutes to separate the single strands. After heating, the samples were immediately cooled on ice and 2 µl of each sample was loaded 20 onto a 6% acrylamide gel containing 89 mM Tris-borate, 2 mM EDTA, pH 8.3 (1 x TBE). The gel was run at 25 W for 4.5 hours at 26°C using 1 x TBE as running buffer. After electrophoresis, the gel was dried and exposed to Kodak X-Omat AR film with an intensifying screen at -70°C for 15 hours.

## 25 Characterization of Polymorphism

Polymorphisms were confirmed by direct sequencing of genomic DNA/PCR products (Fig. 1). One µg genomic DNA was PCR amplified and 1/250 of these products used as template in a second heminested PCR reaction for sequencing. Thirty cycles 30 at 95°C for 1 minute, 58°C for 1 minute, and 72°C for 3 minutes were utilized. PCR products were agarose gel isolated, and 500 ng were directly sequenced using nested oligonucleotides.

- 13 -

**Example 1 - Determination of polymorphism**

Exon 4 of the p53 gene was screened by genomic DNA SSCP analysis as describe above on genomic DNA from 100 individuals. Genomic DNAs were prepared from the peripheral blood of 18 normal individuals; from the peripheral blood, 9 lymphoblastoid lines or fibroblast lines, but not tumor tissue, of 16 patients affected with various cancers; 15 leukemic marrow specimens; 20 neuroblastomas or neuroblastoma cell lines; nine rhabdomyosarcomas or rhabdomyosarcoma cell lines; 10 21 brain tumors; one testicular tumor; and one retinoblastoma cell line. Paired leukemia and brain tumor specimens from one patient with multiple primary cancers were included in the aforementioned samples as reported upon by Perilongo et al., *Leukemia*, 7:912-915 (1993). Matched normal tissues from nine 15 patients with some of the above cancers also were studied.

An identical SSCP pattern difference and silent CCG to CCA change at codon 36 suggesting a p53 polymorphism were found in peripheral bloods of two unrelated normal individuals, matched tumor and normal tissues of a patient with a 20 gangliocytoma/ganglioneuroma, and in a neuroblastoma. The results are depicted in Figure 1. In panel 1, lane 1 is normal peripheral blood, lane 2 is a leukemia cell line, and lanes 3-5 represent brain tumor. In panel 2, lane 1 is a retinoblastoma and lanes 2-9 represent neuroblastomas. In panel 3, lane 1 is 25 a brain tumor, lane 2 is blood from the patient as the sample in lane 1, and lanes 3-5 represent normal tissue samples. T91-84T represents the gangliocytoma/ganglioneuroma specimen. Nb4 represents the neuroblastoma specimen. The normal specimen is designated as such.

30 As can be seen from the figure, four samples out of 100 displayed the polymorphism. All four samples displaying the polymorphism were also heterozygous for the polymorphism. The polymorphism changes a *Fni*I restriction site to a *Bcc*I site. An arrow in Figure 1 represents the pattern of the 35 polymorphic allele. An asterisk indicates the other exon 4 polymorphism at codon 72. The NB4 specimen exhibited both polymorphisms. Other specimens tested were homozygous for the

- 14 -

more common allele. No specimens tested were homozygous for the polymorphism, tumor or normal.

Genomic DNA from the peripheral blood of a normal individual was amplified by PCR and sequenced directly to 5 verify the polymorphism and heterozygosity at codon 36. The results are depicted in Figure 2. As can be seen in Figure 2, there are bands present in the "G" and "A" lanes at the 3' terminal nucleotide of codon 36.

**Example 2 - Determining Genetic Linkage of Unidentified Genes**

10 The polymorphism at codon 36 of p53 can serve as a marker for mapping unidentified genes on chromosome 17 for which loss of heterozygosity has been linked with disease. The region of genomic loss can be mapped using standard techniques and the polymorphism. A recent example of how genetic linkage 15 led to the identification of a new gene is that of the genetic mapping of a locus predisposing to colorectal cancer (Peltomaki et al., *Science*, 260:810-812 (1993), incorporated herein by reference.), where specific chromosomal markers on human chromosome 2 were traced in pedigrees. In this case, the 20 polymorphisms were microsatellites.

**Example 3 - Determining Genetic Linkage of Identified p53 mutation**

Once a tumor possessing a germline mutation is identified, allelic linkage with the polymorphism at codon 36 25 of exon 4 can be assessed. Genomic DNA is extracted from the tumor and the presence of the polymorphism pattern can be screened for using Southern blot analysis. The DNA can be cut with *FinI* and *BccI*. The labelled probe to be used can be p53 cDNA, such as the 1.8 kb *XbaI* p53 cDNA probe derived from clone 30 *php53cl* discussed in Felix et al., *J. Clin. Invest.*, 89:640-647 (1992) (incorporated herein by reference).

Alternatively, if the sample size so requires, genomic DNA is extracted from the tumor and the region of the gene purportedly possessing the mutation and exon 4 can be amplified 35 using PCR and the polymorphism screened for using SSCP. The

particular primers to be used for the PCR amplification will depend upon the location of the mutation in relation to the polymorphism. One skilled in the art could readily determine the appropriate primer to be used from those available in the literature and the sequence of p53 which is available in GenBank. The primers for these purposes would be selected so that the amplified products encompassed both the mutation and the polymorphism. For a list of some primers which can be utilized, see Mitsudomi et al., *supra*.

10 In both instances, the patterns obtained can be compared with genomic DNA from a sample possessing the polymorphism, or the patterns in Figure 1 if testing conditions are the same.

Upon identification of a similar pattern, the 15 amplified PCR product, or genomic DNA region containing the mutation and polymorphism, can be cloned using methods available in the art and the cloned material sequenced. Specifically, in the case of genomic DNA, genomic cloning can be utilized. Briefly, the genomic DNA can be cleaved with 20 restriction enzymes which result in a fragment containing the mutation and the polymorphism. This fragment can then be cloned into a vector with complementary sites. The appropriate restriction enzymes will depend upon the location of the mutation in relation to the polymorphism and can be readily 25 ascertained by one skilled in the art.

The presence of the mutation in the same clone as the polymorphism indicates that both occur on the same allele. Conversely, the presence of one and not the other, suggests that they occur on different alleles.

### 30 Example 4 - Determining Parental Origin

Once allelic linkage has been established between the polymorphism at codon 36 of exon 4 and the germline mutation, genomic DNA can be isolated from tissue from the parents and screened for parental origin of the allele carrying the 35 mutation. The polymorphism can be identified by either Southern blot or SSCP as in Example 3 above.

- 16 -

**Example 5 - Determining Familial Pedigree**

Once allelic linkage has been established between the polymorphism at codon 36 of exon 4 and the germline mutation, genomic DNA can be isolated from tissue from the siblings, 5 offspring, and other relatives of the patient to screen for the presence of the allele carrying the mutation. The polymorphism can be identified by either Southern blot or SSCP as in Example 3 above.

**Example 6 - Determining Loss of Heterozygosity**

10        Regardless whether linkage between the polymorphism at codon 36 of p53 and the germline mutation is established, the presence of absence of heterozygosity for the polymorphism in the patient sample can be ascertained by the methods discussed above. Specifically, heterozygosity for the 15 polymorphism at codon 36 can be ascertained by SSCP or Southern blotting. In the instance of Southern blotting, both *BccI* and *FinI* would be used. Loss of heterozygosity can be ascertained by comparison with normal tissue from the patient.

20        The foregoing examples are meant to illustrate the invention and not to limit it in any way. Those skilled in the art will recognize that modifications can be made which are within the spirit and scope of the invention as set forth in the appended claims.

## SEQUENCE LISTING

## (1) GENERAL INFORMATION:

(i) APPLICANT: Carolyn A. Felix

5 (ii) TITLE OF INVENTION: Polymorphism at Codon 36 of the p53 Gene

(iii) NUMBER OF SEQUENCES: 2

## (iv) CORRESPONDENCE ADDRESS:

(A) ADDRESSEE: Woodcock Washburn Kurtz Mackiewicz &  
15 Morris

(B) STREET: One Liberty Place - 46th Floor

(C) CITY: Philadelphia

(D) STATE: PA

(E) COUNTRY: USA

15 (F) ZIP: 19103

## (v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: DISKETTE, 3.5 INCH, 1.44 Mb STORAGE

(B) COMPUTER: IBM PS/2

(C) OPERATING SYSTEM: PC-DOS

20 (D) SOFTWARE: WORDPERFECT 5.1

## (vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER:

(B) FILING DATE: Concurrently herewith

(C) CLASSIFICATION:

25 (vii) PRIOR APPLICATION DATA:

(A) APPLICATION NUMBER:

(B) FILING DATE:

(viii) ATTORNEY/AGENT INFORMATION:

- 18 -

(A) NAME: Doreen Yatko Trujillo  
(B) REGISTRATION NUMBER: 35,719  
(C) REFERENCE/DOCKET NUMBER: CH-0486

(ix) TELECOMMUNICATION INFORMATION:

5 (A) TELEPHONE: (215) 568-3100  
(B) TELEFAX: (215) 568-3439

(2) INFORMATION FOR SEQ ID NO: 1

(i) SEQUENCE CHARACTERISTICS:

10 (A) LENGTH: 20  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1

15 AGGACCTGGT CCTCTGACTC

20

(2) INFORMATION FOR SEQ ID NO: 2

(i) SEQUENCE CHARACTERISTICS:

20 (A) LENGTH: 20  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2

CGAAGGTACT CTGAAGTTAC

20

- 19 -

What is claimed is:

1. A method for determining linkage of a region of chromosome 17 for which loss of heterozygosity leads to development of disease to a polymorphism at codon 36 of exon 5 4 said method comprising:

- a) isolating genomic DNA corresponding to said region; and
- b) identifying the polymorphism in said DNA.

2. The method of claim 1 wherein the polymorphism 10 is identified by single-strand conformation polymorphism analysis.

3. The method of claim 1 wherein the polymorphism is identified by Southern blotting.

4. The method of claim 1 wherein the disease is 15 cancer.

5. A method for determining genetic linkage of a previously identified germline mutation in the p53 gene resulting in expression of a tumorigenic phenotype with a polymorphism at codon 36 in exon 4 of said gene in a patient 20 extract, said method comprising:

- a) isolating genomic DNA from the tumor;
- b) identifying the polymorphism in said DNA;
- c) cloning the region of the DNA containing the mutation and the polymorphism;
- 25 d) sequencing the cloned DNA; and
- e) ascertaining whether the mutation and polymorphism are present in the same clone.

6. The method of claim 5 wherein the polymorphism is identified using single-strand conformation polymorphism 30 analysis.

- 20 -

7. The method of claim 5 wherein the polymorphism is identified using Southern blotting.

8. The method of claim 5 wherein the patient has been diagnosed with the Li-Fraumeni syndrome.

5 9. A method for determining parental origin of an allele carrying a germline mutation in the p53 gene which has been linked to a polymorphism at codon 36 of exon 4 comprising:

- 10 a) isolating genomic DNA from each parent of a patient possessing the allele; and
- b) identifying the polymorphism in the DNA.

10. The method of claim 9 wherein the polymorphism is identified using single-strand conformation polymorphism analysis.

15 11. The method of claim 9 wherein the polymorphism is identified using Southern blotting.

12. A method for determining familial inheritance of an allele carrying a germline mutation in p53 which has been linked to a polymorphism at codon 36 in exon 4 of the p53 gene said method comprising:

- a) isolating genomic DNA from relatives of a patient possessing the allele;
- b) identifying the polymorphism in said genomic DNA.

25 13. The method of claim 12 wherein the polymorphism is identified using single-strand conformation polymorphism analysis.

14. The method of claim 13 wherein the polymorphism is identified using Southern blotting.

- 21 -

15. A method for determining loss of heterozygosity in chromosome 17 in a tumor sample comprising:

a) isolating genomic DNA from a tumor of a patient exhibiting said tumor;

5 b) identifying a polymorphism at codon 36 of exon 4 in said DNA;

c) determining that the polymorphism is homozygous in said tumor DNA;

10 d) isolating genomic DNA from normal tissue of said patient;

e) identifying the polymorphism in the genomic DNA of said normal tissue; and

f) determining whether said normal tissue is heterozygous for the polymorphism.

15. 16. The method of claim 15 wherein the polymorphism is identified using single-strand conformation polymorphism analysis.

17. The method of claim 15 wherein the polymorphism is identified using Southern blotting.

T91-84T

Nba4

## Normal

A row of four dark, irregularly shaped objects, possibly pieces of debris or charred material, arranged horizontally.

A horizontal strip of dark, irregular shapes on a light background, with arrows at both ends pointing to the right.

FIG. I

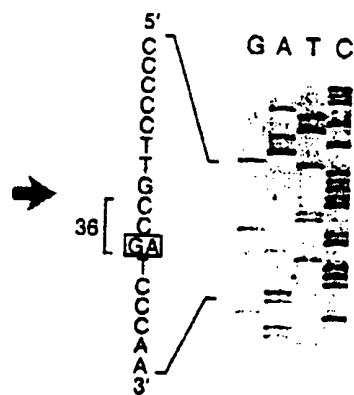


FIG. 2

**SUBSTITUTE SHEET**

## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US95/00158

## A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) C07H 21/04; C10Q 1/66  
US CL 536/24.31; 435/6

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 536/24.31; 435/6

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, MEDLINE, CA

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	ONCOGENE, Volume 5, issued 1990, Chiba et al., "Mutations in the p53 gene are frequent in primary, resected non-small cell cancer," pages 1603-1610, see especially page 1604, Table 1, sample 831.	1-7, 9-17
---		-----
Y	THE JOURNAL OF CLINICAL INVESTIGATION, Vol. 89, issued February 1992, Felix et al., "Hereditary and Acquired p53 Gene Mutations in Childhood Acute Lymphoblastic Leukemia," pages 640-647, see especially the Abstract.	8

<input type="checkbox"/>	Further documents are listed in the continuation of Box C.	<input type="checkbox"/>	See patent family annex.
"A"	Special category of cited documents: document defining the general state of the art which is not considered to be of particular relevance	"T"	Later documents published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"E"	earlier documents published on or after the international filing date	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"L"	documents which may throw doubt on priority claim(s) or which is cited to establish the publication date of another claim(s) or other special reasons (as specified)	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O"	documents referring to an oral disclosure, i.e. exhibitions or other shows	"Z"	document member of the same patent family
"P"	documents published prior to the international filing date but later than the priority date claimed		
Date of the actual completion of the international search		Date of mailing of the international search report	
13 APRIL 1995		28 APR 1995	
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230		Authorized officer SCOTT HOUTTEMAN	
		Telephone No. (703) 308-0196	

Form PCT/ISA/210 (second sheet)(July 1992)